molecular ions activated by photon absorption (ca. 241 eV or 514.5 nm) and CA are comparable.[29]

An interesting aspect of the dynamics of CA processes is the recent study by Kenttamaa and Cooks which shows that low-energy CA deposits more internal energy into the incident ion than does high-energy (keV) CA.[30] Kenttamaa and Cooks' results are totally consistent with our data. That is, at incident ion velocities below the threshold for target gas excitation, CA is relatively efficient, but as the velocity of the incident ion is increased above the threshold for target gas excitation, the favored reaction channel is ionization of the target gas.

Conclusions

Derrick's earlier work and the present work have important implications for collision-induced dissociation/tandem mass spectrometry (CID–TMS) for structural characterization of large molecules. The questions concerning the low efficiency for CID–TMS influence the practical utility of the method,[31] especially at high mass (>3000 daltons) where sensitivity is an overriding consideration. Derrick reported large energy losses accompanying CA, inferring that the principal factors influencing the sensitivity of CID–TMS of large molecular ions are associated with the dynamics of dissociation of the collisionally activated ion. On the other hand, our data (both from the ion beam experiments on the $[M + H]^+$ ion of chlorophyll *a* and the endothermic charge-transfer reactions of small polyatomic ionic systems studied by FTMS) suggest that an important factor influencing the sensitivity of CID–TMS is associated with the CA step. That is, although large energy losses accompany CA, the dominant fraction of the energy loss goes into excitation of the target gas rather than excitation of the incident ion.

The available data suggest that the endothermic charge-transfer reaction is a momentum transfer process. That is, the cross section for charge-transfer reactions should increase as both the velocity and mass of the incident ion increases. Derrick has shown that the energy loss accompanying CA increases linearly with mass of the incident ion, and preliminary FTMS results show that the cross section for endothermic charge transfer increases linearly with velocity of the incident ion. Since the velocity of the incident ion decreases as $m^{-1/2}$, the more critical parameter influencing the efficiency for CID is the mass of the incident ion.

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Registry No. $C_6H_6^+$, 34504-50-2; He, 7440-59-7; Ne, 7440-01-9; Ar, 7440-37-1; Kr, 7439-90-9; chlorophyll *a*, 479-61-8.

(29) Boyd, R. K.; Harris, F. M.; Beynon, J. H. *Int. J. of Mass Spectrom. Ion Proc.* **1985**, *66*, 185.
(30) Kenttamaa, H. I.; Cooks, R. G. *Int. J. Mass Secirom. Ion Phys.* **1985**, *64*, 79.
(31) For discussion of the efficiency for CID see ref 10–12, and McLafferty, F. W.; Bente, P. F.; Kornfield, R.; Tsai, S.-C.; Howe, I. *J. Am. Chem. Soc.* **1973**, *95*, 2120.

The Active Species in Surface-Enhanced Raman Scattering of Flavins on Silver Colloids

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Abstract: The N(3) flavin anion is shown to be the active species in the surface-enhanced Raman (SER) spectra of riboflavin, lumiflavin, FMN, and FAD adsorbed on colloidal silver. Participation of the anion is verified by the pH dependence of the spectra, the near identity of flavin SER spectra in water and deuterium oxide colloids, and the absence of SER for flavins with covalently bound N(3) substituents. The SER spectrum of lumichrome is briefly discussed.

Resonance Raman spectroscopy is now firmly established[1] as a useful tool in the elucidation of the structure of flavins and flavoproteins. For example, resonance Raman spectroscopy can reveal subtle details of the hydrogen bonding interactions in flavoproteins,[2] or charge-transfer interactions between flavins and the substrates or inhibitors of the reactions catalyzed by flavoproteins.[3,4] The technique has also proven useful for identification of the active intermediate in metalloflavoproteins.[5]

The major experimental problem in flavin Raman spectroscopy is the intense fluorescence of the isoalloxazine moiety. The fluorescence is intense enough to completely obscure resonance Raman spectra of free flavins and many flavoproteins. Many approaches to fluorescence rejection and quenching have been applied to flavin Raman spectroscopy.

Tsuboi and Nishimura[6] demonstrated that FMN and FAD Raman spectra could be obtained by excitation into the second or third electronic transition of the isoalloxazine. Spiro and co-workers used coherent Raman spectroscopy to attack the fluorescence problem.[7] Nishina and co-workers[8] relied upon the

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(1) Morris, M. D.; Bienstock, R. J. *Advances in Spectroscopy*; Hester, R. E., Clark, R. J. H., eds.; Wiley: London, Vol. 13, in press.
(2) Bienstock, R. J.; Schopfer, L. M.; Morris, M. D. *J. Am. Chem. Soc.* **1986**, *108*, 1833–1838.
(3) Williamson, G.; Engel, P. C.; Nishina, Y.; Shiga, K. *FEBS Lett.* **1982** 29–32.
(4) Schmidt, J.; Reinsch, J.; McFarland, J. T. *J. Biol. Chem.* **1981**, *256*, 11667–11670.
(5) Sugiyama, T.; Nisimoto, Y.; Mason, H. S.; Loehr, T. M. *Biochemistry* **1985**, *24*, 3012–3019.
(6) Nishimura, Y.; Tsuboi, M. *Chem. Phys. Lett.* **1978**, *59*, 210–213.
(7) Dutta, P. K.; Nestor, J. R.; Spiro, T. G. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 4146–4149.

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use of fluorescence-quenching proteins to obtain flavin Raman spectra. McFarland and co-workers demonstrated that potassium iodide and other collisional quenchers could be used to obtain flavin Raman spectra.[9] Recently, Sugiyama and co-workers have demonstrated that, for moderately fluorescent flavoproteins, signal averaging and background subtraction can be an effective strategy.[5] Each of these experimental techniques has proven valuable.

Copeland and co-workers[10] have demonstrated that surface-enhanced Raman spectroscopy (SERS) on colloidal silver can be used to obtain high quality spectra of flavoproteins. The combination of fluorescence quenching and Raman intensity enhancement provided by the silver surface allows acquisition of spectra at submicromolar levels. Relative band intensities are perturbed from the ranges normal in flavin Raman spectroscopy, and some bands appear at frequencies 5–30 cm^{-1} different from their usual positions.

The work of Copeland et al. suggests that SERS may assume an important role in flavin Raman spectroscopy but also that careful definition of the interaction of flavins with a silver surface is necessary for exploitation of flavin SERS. The present study addresses the question of the nature of the active species in the SERS of free flavins and flavoproteins.

Experimental Section

Riboflavin, FMN, and FAD were purchased from Sigma. FMN was purified[11] by adsorption onto an apo-flavodoxin column, followed by elution and concentration. Lumiflavin, N(3)-methyl lumiflavin, N(3)-carboxymethyl lumiflavin, N(3)-methyl riboflavin, and lumichrome were generous gifts from Dr. Peter Hemmerich, University of Konstanz, W. Germany. All other reagents were ACS reagent grade and were used as received. Type I water was used for preparation of all H_2O solutions. For deuteration experiments, 99.8% D_2O (Cambridge Isotope Laboratories) was used as received. All glassware was cleaned in concentrated nitric acid prior to use.

Silver colloids were prepared by a variant of the standard[12] EDTA reduction procedure. To 100 mL of boiling water were added 1.0 mL of 0.1 *M* EDTA and 4.0 mL of 0.1 *M* NaOH followed by 1.3 mL of 0.1 *M* $AgNO_3$. After boiling for about 60 s, 0.3 mL of 0.1 *M* HCl was added. The mixture was then kept at boiling for 2–3 min until an intense yellow-brown color developed. Solutions prepared by this technique were used if they exhibited an absorption maximum at 414 nm. Solutions were ready for use immediately after preparation.

Silver solutions in D_2O were prepared by the citrate reduction method.[13] To 20 mL of boiling D_2O in a reflux system equipped with a $CaCl_2$ trap was added 6 mg of sodium citrate. To this system was added 10 mL of a 0.003 *M* $AgNO_3$ solution in D_2O. The system was refluxed for about 1 h. The resulting solution was characterized by a yellow-brownish color, absorption maximum 412 nm. Solutions prepared by this technique were aged for 2 weeks before use.

In general, solutions were not buffered except by the buffering capacity of the citrate or EDTA remaining in the solution dispersion. The pH of the citrate solutions was always 7.2–7.3. The pH of the EDTA solutions was brought into this range by addition of HNO_3 as necessary.

Raman spectra were obtained with argon ion 488-nm excitation. Laser power was limited to 15–20 mW at the sample. Either a scanned system with photon counting (Spex 1401 monochromator, RCA C31034 photomultiplier) or a spectrograph with array detector (Spex 1877 spectrograph, Tracor Northern 6122 intensified diode array) was used to obtain spectra. Scanned spectra were acquired at 100 cm^{-1}/min with 9-cm^{-1} resolution. Multichannel spectra were acquired with 1–10 s exposure time at 4-cm^{-1} resolution. In all experiments, samples were contained in standard melting point capillary tubes.

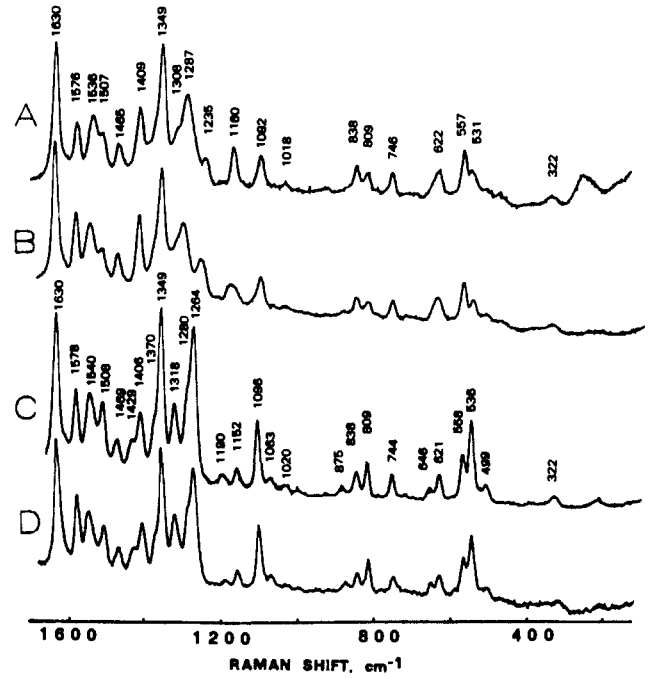


Figure 1. SER spectra of 1 × 10^{-6} *M* riboflavin and lumiflavin on colloidal silver: A, riboflavin in H_2O; B, riboflavin in D_2O; C, lumiflavin in H_2O; D, lumiflavin in D_2O.

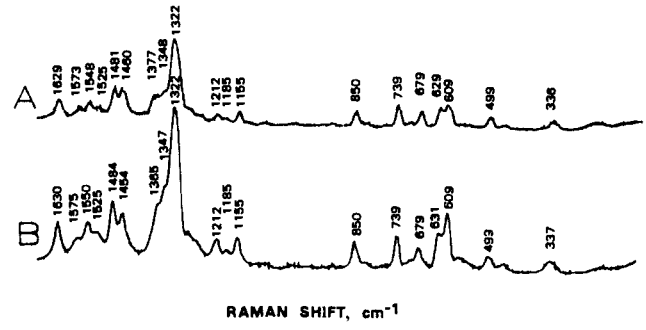


Figure 2. SER spectra of 1 × 10^{-6} *M* lumichrome on colloidal silver, pH 7.2; A, H_2O solution; B, D_2O solution.

Absorption spectra were obtained on Varian/Cary 219 spectrophotometer.

Results and Discussion

Silver solutions prepared by both EDTA reduction and by citrate reduction show the same flavin Raman enhancement. Because the solutions prepared by EDTA reduction could be used without aging and remained stable for more than 3 months, they were generally preferred. However, the EDTA procedure occasionally yielded unsuitable solutions. As an economy measure, the unfailingly reliable citrate procedure was used to prepare solutions in D_2O solution.

SER spectra of RF and LF in both H_2O and D_2O are shown in Figure 1. At 1 × 10^{-6} *M* concentrations, intense spectra were obtained, with no discernible fluorescence backgrounds.

The SERS frequencies for riboflavin are presented in Table I. For comparison purposes, we include resonance Raman data[9] for RF using potassium iodide as a collisional quencher, for RF and N(3)-methyl RF in egg white riboflavin binding protein,[8] and for the N(3) anion of FAD.[14] Table II contains our data for lumiflavin and the equivalent resonance Raman data for LF and N(3)-methyl LF.[9,15] No resonance Raman data are available for the N(3) anion of lumiflavin.

(8) Nishina, Y.; Kitagawa, T. Y.; Shiga, K.; Horiike, K.; Matsumura, Y.; Matsui, K.; Watari, H.; Yamano, T. *J. Biochem. (Tokyo)* **1978**, *84*, 925–932.
(9) Schmidt, J.; Coudron, P.; Thompson, A. W.; Watters, K. L.; McFarland, J. T. *Biochemistry* **1983**, *22*, 76–84.
(10) Copeland, R. A.; Fodor, S. P. A.; Spiro, T. G. *J. Am. Chem. Soc.* **1984**, *106*, 3872–3874.
(11) Mayhew, S. G.; Strating, M. J. J. *Eur. J. Biochem.* **1975**, *59*, 539–544.
(12) (a) Gantner, E.; Steinert, D.; Reinhardt, J. *Anal. Chem.* **1985**, *57*, 1658–1662. (b) Fabrikanos, V. A.; Athanassiou, S.; Lieser, K. H. *Z. Naturforsch., B: Anorg. Chem., Org. Chem., Biochem., Biophys., Biol.* **1963**, *188*, 612–617.
(13) (a) Lee, P. C.; Meisel, D. J. *J. Phys. Chem.* **1982**, *86*, 3391–3395. (b) Hildebrant, P.; Stockburger, M. *J. Phys. Chem.* **1984**, *88*, 5935–5944.
(14) Dutta, P. K.; Spencer, R.; Walsh, C.; Spiro, T. G. *Biochim. Biophys. Acta* **1980**, *623*, 77–83.
(15) Nishina, Y.; Shiga, K.; Horiike, K.; Tojo, H.; Kasai, S.; Yanase, K.; Matsui, K.; Watari, H.; Yamano, T. *J. Biochem. (Tokyo)* **1980**, *88*, 403–409.

Table I. Observed Frequencies (cm^{-1}) in SERS in RF and 3-D RF, in RRS of RF, 3-D RF, 3-Methyl RF, and 3-Anion FAD in Different Conditions

bands[a]	SER RF	SER 3-D RF	RR RF[b]	RR RF[c]	RR 3-D RF[b]	RR 3-D RF[c]	RR 3-Me RF[c]	RR anion FAD[d]
I	1630	1630	1629	1632	1629	1632	1633	1629
II	1576	1577	1582	1584	1581	1585	1586	1578
III	1536	1536	1547	1547	1549	1553	1556	na
IV	1507	1507	1501	1503	1501	1504	1511	1505
V	1465	1465	1462	1465	1462	1463	1465	na
	1420 (sh)	1420 (sh)	no	no	sh	1431	1420	na
VI	1409	1409	1408	1407	1407	1408	1403	1406
	1370 (sh)	1370 (sh)	no	no	sh	sh	sh	na
VII	1349	1350	1353	1355	1352	1352	1355	1346
VIII	1308 (sh)	1309 (sh)	no	1302	sh	sh	no	na
IX	no	no	1277	1284	1270 (sh)	sh	1275	na
X	1287	1289	1256	1253	1293	1296	1289	na
	1265 (sh)	sh	no	no	no	no	no	no
XI	1235	1246	1228	1230	1232	1235	1231	1232
	no	no	no	1213	no	1212	1212	na
XII	1190 (w)	1190 (w)	1182	1180	1210	1181	no	1188
XIII	1160	1163	1157	1160	1163	1151	1158	1155
	1092	1090	no	1101	1141	1139	1138	na
	1018	1020	na	1071	na	1042	weak	na
	no	no	na	994	na	987	994	na
	838	835	na	834	na	830	830	na
	809	808	na	789	na	772	no	na
	746	740	na	740	na	742	731	na
	622	624	na	na	na	na	na	na
	557	557	na	na	na	na	na	na
	531	532	na	na	na	na	na	na
	496	497	na	na	na	na	na	na
	322	326	na	na	na	na	na	na

[a] Numbers of Bowman and Spiro *Biochemistry* **1981**, *20*, 3313–3318. [b] Reference 9. [c] Reference 8. [d] Reference 14. (sh) shoulder, (no) not observed, (na) not available, (w) weak.

Table II. Observed Frequencies (cm^{-1}) in SERS of LF and 3-D LF, in RRS of LF, 3-D LF, and 3-Methyl LF in Different Conditions

bands[a]	SER LF	SER 3-D LF	RR LF[b]	RR LF[c]	RR 3-D LF[b]	RR 3-D LF[c]	RR 3-Me LF[c]
I	1630	1631	1628	1630	1629	1630	1628
II	1578	1579	1584	1585	1583	1583	1584
III	1540	1546	1551	1553	1556	1555	1550
IV	1508	1508	1503	1503	1503	1503	1505
V	1469	1470	1465	1465	1462	1462	1462
	1429 (sh)	1429 (sh)	sh	weak	sh	no	no
VI	1406	1408	1405	1406	1405	1403	1394
	1370 (sh)	1370 (sh)	no	sh	sh	no	no
VII	1349	1349	1353	1354	1353	1352	1353
VIII	1318	1319	no	no	1306	no	no
IX	1280 (sh)	1282 (sh)	1278	weak	1282	weak	1285
X	1264	1266	1243	1243	1268	1262	1266
XII	1190	1190	no	no	1196	1204	1207
XIII	1152	1152	1147	1150	1142	1140	1173
	1096	1096	1084	1085	1078	1084	1090
	1063	1063	na	no	na	no	no
	1020	1020	na	993	na	986	no
	920 (w)	915 (w)	na	no	na	no	no
	875	870	na	880	na	858	841
	838	838	na	835	na	833	827
	809	809	na	790	na	770	no
	744	744	na	741	na	736	732
	646	646	na	638	na	636	no
	621	622	na	608	na	606	593
	558	558	na	no	na	no	no
	536	536	na	no	na	524	525
	499	500	na	527	na	499	499
	no	no	na	418	na	415	na
	322	326	na	no	na	na	na

[a] Numbers of Bowman and Spiro, *Biochemistry* **1981**, *20*, 3313–3318. [b] Reference 9. [c] Reference 15. (sh) shoulder, (no) not observed, (na) not available, (w) weak.

Riboflavin can undergo photochemical reaction to lumiflavin and to lumichrome.[16] We obtained exactly the same spectra for RF by using multichannel array detection with laser exposure of 2–3 s and with scanned spectra acquired over a period of 10–15 min. Control experiments with the sample in a pumped system verified lack of photochemical reaction of the time scale of our experiments. With prolonged laser exposure (1 h or more), a lumiflavin spectrum can be observed in a riboflavin sample. Ultimately, a lumichrome spectrum, identical with that in Figure 2, can be obtained, but not until the onset of colloid coagulation, as evidenced by formation of visible black silver particles. Under the conditions of our experiments, valid SER spectra of the reported flavins are obtained.

(16) Beinert, H. In *The Enzymes*, 2nd ed.; Boyer, P. D., Lardy, M., Myrback, K., Eds.; Academic Press: New York, 1960; Vol. 2A, pp 339–417.

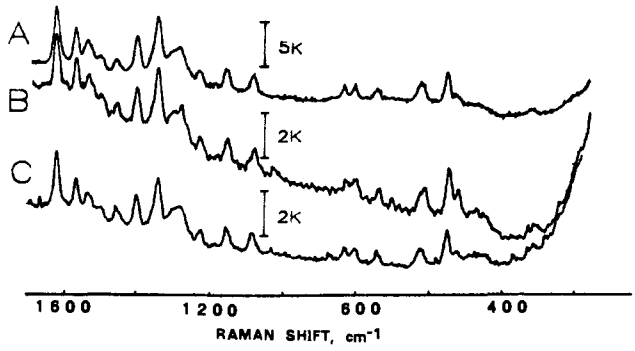


Figure 3. SER spectra of riboflavin and the nucleotides, 1×10^{-6} *M*, pH 7.2: A, riboflavin; B, FMN; C, FAD.

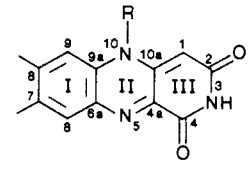
The SER spectra of aqueous solutions of 1×10^{-6} *M* FMN and FAD are presented in Figure 3. The SER spectra of FMN and FAD are qualitatively similar to the SER spectrum of riboflavin. There are no changes which might be attributed to formation of intramolecular complexes or the presence of anionic phosphate. In this, the molecules parallel their behavior in resonance Raman spectroscopy. Quantitatively, the three flavins seem to be somewhat different.

We have verified that the spectrum of riboflavin is not perturbed by the presence of excess adenine or excess 5-adenosine monophosphate. If adenine is present (5×10^{-6} *M*), its intense 738-cm^{-1} band is visible at about the same intensity as the stronger RF bands, at pH 7.2. However, excess (10×10^{-6} *M*) 5'-AMP does not result in a visible adenine band at the same pH values. We attribute the differences to strong silver–phosphate binding, which would keep the adenine away from the silver surface.

At 10^{-6} *M*, riboflavin SER spectra are always more intense than FMN spectra, which in turn are more intense than FAD spectra. The intensity differences are most pronounced immediately after preparing the samples. The signal intensity increases if a stationary sample is irradiated by the laser for 5–10 min. There are no changes in band positions or relative intensities. These effects are more pronounced in FMN and FAD than in RF or LF. If the flavin/Ag solution mixtures are allowed to stand overnight in the dark at room temperature, the intensity of the FMN and FAD samples increases to within a factor of two of that for the riboflavin sample. At 1×10^{-7} *M*, FMN and FAD spectra are initially at the same intensity, while RF spectra are only twice as intense. The most probable explanation for the differences in riboflavin, FMN and FAD intensities is that the compounds cause time-dependent changes in the colloids. These changes appear to be faster with riboflavin than with the other flavins.

In repeated attempts we have been unable to obtain SER spectra for N(3)-substituted riboflavins or lumiflavins. No bands were obtained for N(3)-methyl riboflavin, N(3)-methyl lumiflavin, or N(3)-carboxymethyl lumiflavin. Control experiments verified that the solution preparations gave the usual SER spectra of the parent compound at the expected intensity. We could obtain riboflavin or lumiflavin SER spectra with the same colloids at concentrations of 1×10^{-8} *M* or below with good signal/noise ratio. SER signals (without substantial fluorescence from uncomplexed flavin) at such low flavin concentrations indicate that the complex between flavin and silver is quite tight. The dissociation constant may be estimated to be about 10^{-8} *M* or less. We conclude that N(3)-methyl flavins are not SERS active on silver colloids and suggest the involvement of the N(3) position in the flavin/colloid interaction.

For both riboflavin and lumiflavin, the SER spectra are different from the resonance Raman spectra in bands II, III, VII, and X. The ^{13}C and ^{15}N substitution studies of Kitagawa and co-workers[17] demonstrate that these bands have major contributions from C(4a)–N(5), C(4a)–C(10a), and N(3)–C(4) stretches (see **1** for ring numbering). There is little C(2)–N(3) stretching content. Similar conclusions can be reached from the spectra of deazaflavins[14] and C(2)-thio FMN.[18] These observations implicate the N(3) position in the flavin/colloid interaction, and further they suggest the involvement of C(4)=O and possibly N(5). Absence of effects on bands sensitive to C(2) argues against the involvement of this position.



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The complete absence of SERS from 3-substituted riboflavin and lumiflavin could arise in either of two ways. First, the presence of a bulky group at position 3 could block access to the colloid surface. Second, the active species could be the deprotonated flavin, which could not form from the covalently substituted derivatives.

There does not appear to be any general impediment to observation of SERS of tertiary amines or other compounds containing bulky substituents on nitrogen. For example, Garrell and Schultz[19] have obtained good SERS spectra of *tert*-butylamine on colloidal silver in acetonitrile solutions. Heard and co-workers have demonstrated strong SERS spectra of cetylpyridinium and cetylquinolinium salts on colloidal silver.[20] However, to distinguish between the steric effect and ionization hypotheses, we measured the pH dependence of the spectra and the effects of substitution of deuterium for the labile proton at N(3).

Mixing flavin with D_2O under neutral conditions causes exhange of the proton at position N(3) for a deuteron. Deuteration in this fashion produces surprisingly little change in the SER spectra of riboflavin, lumiflavin, or lumichrome. In the resonance Raman spectra of riboflavin and lumiflavin, bands X and XIII are sensitive to deuteration.[9,17] In the resonance Raman spectrum of riboflavin band X is a strong band found about 1255 cm^{-1}. Band X normally shifts about 40 cm^{-1} to higher frequency. Band XIII shifts about 10 cm^{-1} to lower frequency, and the two bands in this region become more clearly separated.

We assign the strong RF SERS band at 1287 cm^{-1} as band X. This frequency is slightly lower than observed in the resonance Raman spectra of deuterated riboflavin, and almost coincident with the position of band X in 3-methylriboflavin. Deuteration shifts the SERS frequency only 2 cm^{-1}. Similarly, we assign the strong 1264 cm^{-1} LF SERS band as band X. Again, this is essentially coincident with the resonance Raman frequency of band X in deuterated lumiflavin and 3-methyllumiflavin. Deuteration shifts band X of LF SERS only 2 cm^{-1} to higher frequency. Band XIII is shifted from 1235 to 1246 cm^{-1} upon deuteration. This shift is larger than the 4–5 cm^{-1} observed in resonance Raman spectra. Band III is shifted 6 cm^{-1} to higher frequency in LF SERS, about the same as the band shift in LF resonance Raman spectra.

Deuteration has a negligible effect (2 cm^{-1} or less) on all other bands in the LF and RF SER spectra, as is the case in resonance Raman spectra. This reduced sensitivity of the SERS spectra to deuteration suggests that the exchangeable proton at N(3) is not present in the flavin/colloid complex.

The deuterium shifts observed in band XI of the riboflavin SER spectrum and band III of the lumiflavin spectrum may reflect differences in hydrogen bonding to N(1), C(2)=O, and C(4)=O. Band XI, for example, is known to be sensitive to ^{13}C substitution at positions 4 and 4a and contains contributions from N(3)–C(4) stretching. The shift in lumiflavin band III upon deuteration is also observed in its resonance Raman spectrum.[9,17] However,

(17) Kitagawa, T.; Nishina, Y.; Koyogu, Y.; Yamano, T.; Ohishi, N.; Takai-Suzuki, A.; Yagi, K. *Biochemistry*, **1979**, *18*, 1804–1808.

(18) Irwin, R. M.; Visser, A. J. W. G.; Lee, J.; Carreira, L. A. *Biochemistry* **1980**, *19*, 4639–4646.

(19) Garrell, R. L.; Schultz, R. H. *J. Colloid. Interface Sci.* **1985**, *105*, 483–491.

(20) Heard, S. M.; Grieser, F.; Barraclough, C. G. *Chem. Phys. Lett.* **1983**, *95*, 154–158.

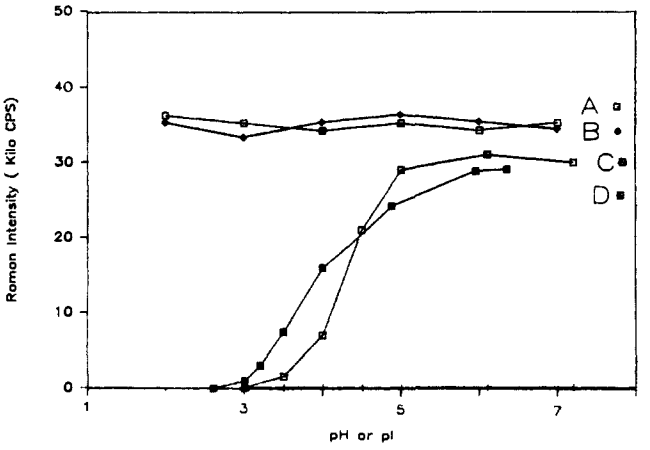

Figure 4. The pH and ion concentration dependence of riboflavin and lumiflavin SER 1630 cm^{-1} intensity: A, riboflavin pNaOH dependence; B, riboflavin $pNaNO_3$ dependence; C, riboflavin pH dependence; D, lumiflavin pH dependence.

deuteration does not affect this band in the resonance Raman spectrum of riboflavin.[9,17]

Figure 4 shows the effect of changing pH or ionic strength on the SER spectrum of riboflavin. We plot only band I intensities. There are no changes in the relative intensities of the various bands in the spectra. Inspection of the figure shows that there is no effect upon addition of hydroxide, up to pH 11.2. This is above the pK_a (10) of the N(3) position.[21] However, making the solution acidic produces gradual attenuation, with an abrupt loss of signal around pH 4. Ionic strength controls with $NaNO_3$ demonstrate that there is no ionic strength effect until above $pI = 3$. At this ionic strength, the colloid is dispersed, and the spectrum disappears. Similar effects are produced by addition of HCl or NaCl, except that the colloid is destabilized at lower ionic strengths than in nitrate solutions. LF shows the same behavior as RF. A pH behavior such as this is consistent with the N(3) position being unprotonated in the complex. One would predict that a tight complex between the N(3) anion and the silver colloid would reduce the apparent pK_a of N(3). Furthermore, protonation at N(3) would disrupt the complex, causing loss of the signal, as observed.

Alternatively, the pH dependence could be a result of changes in the colloid morphology. Siiman and Lepp[22] observed that colloids prepared by the citrate reduction procedure coagulated within 10 min at pH 4.4 or lower. However, we find that colloids prepared by the EDTA procedure are stable for at least 1 h at pH 3 and for several hours at pH 4. These findings are consistent with the acid/base properties of citric acid and EDTA. Thus changes of colloid morphology would be unlikely to be the cause of the pH dependence shown in Figure 4.

Comparison of the resonance Raman spectra of riboflavin and lumiflavin and their N(3)-methyl derivatives (Table I and II) shows that there are no unusual effects caused by the methyl substitution.[8,9,15] The N(3)-methyl compounds produce spectra quite similar to those produced by deuterium substitution at the N(3)-position. The effects are completely accounted for by removal of the coupling between N–H bending and C–N stretching, which contribute to the vibrations causing bands X and XIII.

The pH dependence of the spectra, the absence of signals from N(3)-substituted compounds, and the small deuterium isotope effects all point to the N(3)-anion as the active species in the SERS of the free flavins. Further support for this conclusion is obtained from the resonance Raman spectrum of the N(3) anion of FAD.[14] In this system (Table I), band II is shifted 4–6 cm^{-1} to lower frequency compared to neutral free flavins. In the SER spectra, we observe a similar shift in band II to 1576 cm^{-1}. Copeland and co-workers report similar frequencies for band II in riboflavin/RBP and glucose oxidase SERS.

We cannot establish conclusively whether the flavin is oriented parallel to the silver surface or at an angle to it. However, if flavins were adsorbed parallel to the surface, then one would expect to see SERS from neutral molecules, including the N(3)-substituted derivatives. The absence of these spectra argues strongly for a noncoplanar orientation.

A superficially similar pattern of band shifts is observed in the resonance Raman spectra of the metal ion complexes[23,24] where metal binding is to N(5) and C(4)=O. However, in the metal ion complexes, band I is shifted 5–10 cm^{-1} to lower wavenumbers, and band II is shifted at least 10 cm^{-1} lower. Because we see no shift in band I, we can confirm the suggestion of Copeland et al.[10] that binding to N(5)—C(4)=O is not the cause of the perturbations observed in the flavin SER spectra.

In summary, the weight of the experimental evidence is that the flavin is strongly bound to colloidal silver through N(3), as suggested by Copeland et al.[10] and that the bound species is anionic.

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Registry No. N(3)-methyl RF, 28721-76-8; N(3)-methyl LF, 18636-32-3; FMN, 146-17-8; FAD, 146-14-5; silver, 7440-22-4; riboflavin, 83-88-5; lumiflavin, 1088-56-8; lumichrome, 1086-80-2.

(21) Draper, R. D.; Ingram, L. L. *Arch. Biochem. Biophys.* **1968**, *125*, 802–808.
(22) Siiman, O.; Lepp, A. *J. Phyl. Chem.* **1984**, *88*, 2641–2650.
(23) Benecky, M.; Yu, J.-J.; Watters, K. L.; McFarland, J. T. *Biochim. Biophys. Acta* **1980**, *626*, 197–207.
(24) Benecky, M. J.; Dowling, M. G.; Clarke, M. J.; Spiro, T. G. *Inorg. Chem.* **1984**, *23*, 865–869.